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An Expanded Plastid Phylogeny of *Marsilea* with Emphasis on North American Species

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Abstract.—Ferns of the genus *Marsilea* (water clover) are potentially invasive aquatic and wetland plants. They are difficult to identify to species because of subtle diagnostic characters, the sterile condition of many specimens, and unresolved taxonomic problems. We sequenced four plastid regions (*rbcL*, *rps4*, *rps4-trnS* spacer, and *trnL-F* spacer) from 223 accessions across ca. 38 species. Our goals were to: 1) attempt to identify problematic *Marsilea* specimens from the southeastern U.S., and 2) assess species delimitation using molecular data. Florida specimens previously identified as *M.* aff. *oligospora* do not match true *M. oligospora* (native to the western USA), and might represent an undescribed native species. The molecular data fail to resolve many species as monophyletic within the New World *Marsilea* section *Nodorhizae*. The data reveal two strongly supported clades within section *Nodorhizae*: 1) A western U.S. /Mexican clade; and 2) A U.S. Gulf coastal plain/Florida/Caribbean clade. This DNA/morphology discordance suggests that these taxa either may have hybridized extensively or that the number of *Marsilea* species within these clades may be overestimated. Either case warrants the addition of nuclear data sets and reevaluation of the species boundaries within the genus.

Key Words.—*Marsilea*, phylogenetics, plastid, species delimitations

Marsilea L. (ca. 50 spp.) occur worldwide as two ecological types: 1) true aquatic species with glabrous leaves and fleshy rhizomes that inhabit more permanent water bodies, and 2) semi-aquatic species with hairy leaves and tough, fibrous rhizomes that prefer fluctuating wetland habitats and prevail through seasonal extremes in wet and dry periods (Jacono and Johnson, 2006). *Marsilea* have few dependable morphological characters on which to base species-level identifications. Phenotypic plasticity is widespread, and sporocarps, which contain many characters used for species delimitation, are commonly absent in field populations. Because identification of *Marsilea* based upon morphology is so difficult, molecular data might provide more reliable tools for identification.

The impetus for this study was an applied resource management need to clarify the identity of three western North American species of *Marsilea* in Florida (Jacono and Johnson, 2006). *Marsilea vestita* Hook. & Grev. and *M. macropoda* Engelm. ex A. Braun have been regarded as introduced to eastern

North America based on their disjunct and widely scattered populations at ruderal sites in Gulf coastal Alabama and Florida. A third species, centered on three central Florida counties, was tentatively identified as *M.* aff. *oligospora* Goodd. (Jacono and Johnson, 2006) based on sporocarp morphology; however, *Marsilea oligospora* is a semi-aquatic North American species otherwise endemic to the northern fringe of the Great Basin. Variation was noted between the Florida and the Great Basin material and it was difficult for the authors to speculate how a geographically restricted plant with no known economic value might have become established in central Florida over 100 years ago. The great difference in climate between northwestern U.S. and Florida added to our suspicion that these were two different taxa. These Florida *M.* aff. *oligospora* were first collected in the early 1890s near Eustis, Florida, and their determination has vacillated from *M. vestita*, an introduction from the western U.S. (Ward and Hall, 1976) to *M. ancylopoda* A.Braun, a rare and potentially extinct native species (FNA, 1993).

Here we use DNA sequences of four plastid regions (*rbcL*, *rps4*, the *rps4-trnS* spacer, and the *trnL-F* spacer) to expand upon the recent molecular phylogeny of *Marsilea* (Nagalingum *et al.*, 2007), using a greater sampling of North American specimens. Our first objective was to determine the status of the Florida plants assigned to *M.* aff. *oligospora*. We surveyed all known populations of *Marsilea* within Florida and compared them to all U.S., Mexican, and Caribbean species, as well as *Marsilea* species common in the aquatic plant trade that are established in the southeastern U.S. These data will provide a baseline for evaluating *M.* aff. *oligospora* in Florida and for distinguishing future introductions of *Marsilea*. Our second objective is to assess species monophyly using multiple accessions of each species, particularly for the North American specimens assigned to *Marsilea* sect. *Nodorhizae*.

Materials and Methods

Thirty-three samples were included from Nagalingum *et al.* (2007), and are distinguished by the GenBank prefix DQ; the remainder were generated in this study (Table 1). Because Florida collections of *M. oligospora* were hypothesized to be introductions from the western U.S. (Jacono and Johnson, 2006), we included as many specimens as possible from western states. Species not present in the Nagalingum *et al.* (2007) study include *M. coromandelina* Willd., *M. costulifera* D.L.Jones, *M. crenulata* Desv., *M. deflexa* A.Braun, *M. exarata* A.Braun, *M. fournieri* C.Chr., *M. hirsuta* R.Br., *M. mexicana* A.Braun, *M. mucronata* A.Braun, *M. scalaripes* D.M. Johnson, *M. tenuifolia* Engelm. ex Kunze, and *M. uncinata* A.Braun.

Samples were taken from herbarium specimens. Leaf samples (ca. 25 mm^2) were ground using a tissue mill and extracted using a modified version of the 2× CTAB procedure of Doyle and Doyle (1987) with exclusion of beta-mercaptoethanol and inclusion of 5 units of proteinase K. Primers for *rbcL* were designed to allow amplification and sequencing in two overlapping

TABLE 1. Specimens sampled for DNA analysis in this study and corresponding GenBank numbers. DNA numbers (column 1) correspond to three-digit numbers following taxon names in Fig. 1. DNA numbers 256–280 are data from Nagalingum *et al.* (2007); the remainder were generated in this study. USA localities include state abbreviation followed by county.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
005	*Marsilea ancylopoda* A. Braun	FJ533947	FJ533854	FJ534040	FLAS	E. Lott 3321	Mexico: Jalisco
006	*Marsilea hirsuta* R. Br.	FJ533948	FJ533855	FJ534041	FLAS	Beckner 2619	USA: FL: Pinellas
007	*Marsilea macropoda* Engelm. ex A. Braun	FJ533956	FJ533864	na	FLAS	Ward 7623	USA: TX: San Patricio
008	*Marsilea hirsuta* R. Br.	FJ533953	FJ533861	FJ534046	FLAS	Jacono 257	USA: FL: Hillsborough
009	*Marsilea hirsuta* R. Br.	FJ533954	FJ533862	FJ534047	FLAS	Jacono 235	USA: FL : Pinellas
010	*Marsilea macropoda* Engelm. ex A. Braun	FJ533957	FJ533865	FJ534049	FLAS	Burkhalter 8846	USA: AL: Mobile
011	*Marsilea macropoda* Engelm. ex A. Braun	FJ533958	FJ533866	FJ534050	FLAS	Burkhalter 5672	USA: AL: Mobile
012	*Marsilea macropoda* Engelm. ex A. Braun	FJ533959	FJ533867	FJ534051	FLAS	McAlpin s.n.	USA: FL: Sarasota
013	*Marsilea ancylopoda* A. Braun	HQ631073	HQ631266	HQ631171	FLAS	E. Lott 3321	Mexico: Jalisco
014	*Marsilea minuta* L.	FJ533965	FJ533873	FJ534057	FLAS	J. Allison 8001	USA: GA: Cobb
015	*Marsilea minuta* L.	FJ533966	FJ533874	FJ534058	FLAS	J. Germann s.n.	USA: GA: McDuffie
016	*Marsilea minuta* L.	FJ533967	FJ533875	FJ534059	FLAS	Jacono 394	USA: FL: Escambia
017	*Marsilea minuta* L.	FJ533968	FJ533876	FJ534060	FLAS	Burkhalter 13304	USA: FL: Escambia
018	*Marsilea minuta* L.	FJ533969	FJ533877	FJ534061	FLAS	Jacono 498	USA: FL: St. Lucie
020	*Marsilea mutica* Mett.	HQ631074	HQ631267	FJ534075	FLAS	Jacono 503	USA: OK: Hughes
021	*Marsilea mutica* Mett.	FJ533983	FJ533891	FJ534076	FLAS	Jacono 362	USA: GA: Dekalb
022	*Marsilea mutica* Mett.	FJ533984	FJ533892	FJ534077	FLAS	Cooper s.n.	USA: GA: Fulton
023	*Marsilea mutica* Mett.	FJ533985	FJ533893	FJ534078	FLAS	Jacono 361	USA: GA: Spalding
024	*Marsilea mutica* Mett.	HQ631075	HQ631268	HQ631172	FLAS	Kirk s.n.	USA: MS: Madison
025	*Marsilea mutica* Mett.	FJ533986	FJ533894	FJ534079	FLAS	Davis 1208	USA: FL: Alachua
026	*Marsilea mutica* Mett.	FJ533987	FJ533895	FJ534080	FLAS	Rodgers s.n.	USA: FL: Hillsborough
027	*Marsilea mutica* Mett.	FJ533988	FJ533896	FJ534081	FLAS	Greene s.n.	USA: AL: Marion
028	*Marsilea mutica* Mett.	FJ533989	FJ533897	FJ534082	FLAS	Jacono 502	USA: AL: Marion
030	*Marsilea* aff. *oligospora* Goodd.	FJ533993	FJ533901	FJ534086	FLAS	Jacono 508	USA: FL: Lake
031	*Marsilea* aff. *oligospora* Goodd.	FJ533994	FJ533902	FJ534087	FLAS	Jacono 510	USA: FL: Lake
032	*Marsilea* aff. *oligospora* Goodd.	FJ533995	FJ533903	FJ534088	FLAS	Jacono 511	USA: FL: Lake
033	*Marsilea* aff. *oligospora* Goodd.	FJ533996	FJ533904	FJ534089	FLAS	Jacono 571	USA: FL: Lake
034	*Marsilea* aff. *oligospora* Goodd.	FJ533997	FJ533905	FJ534090	FLAS	Jacono 572	USA: FL: Lake

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
035	*Marsilea* aff. *oligospora* Goodd.	FJ533998	FJ533906	FJ534091	FLAS	Brinson s.n.	USA: FL: Seminole
036	*Marsilea* aff. *oligospora* Goodd.	HQ631076	HQ631269	FJ534092	FLAS	Jacono 177	USA: FL: Seminole
037	*Marsilea* aff. *oligospora* Goodd.	HQ631077	FJ533907	FJ534093	FLAS	Meisenburg s.n.	USA: FL: Volusia
038	*Marsilea vestita* Hook. & Grev.	FJ534005	FJ533913	FJ534098	FLAS	Raymond & Painter 81	USA: LA: Caldwell
039	*Marsilea vestita* Hook. & Grev.	FJ534028	FJ533935	FJ534122	FLAS	Thomas 114,754	USA: LA: Caldwell
040	*Marsilea vestita* Hook. & Grev.	FJ534007	FJ533915	FJ534100	FLAS	Brodie s.n.	USA: FL: Collier
041	*Marsilea vestita* Hook. & Grev.	FJ534008	FJ533916	FJ534101	FLAS	Jacono 619	USA: FL: Collier
042	*Marsilea vestita* Hook. & Grev.	FJ534009	FJ533917	FJ534102	FLAS	Anderson 7356	USA: FL: Franklin
044	*Marsilea vestita* Hook. & Grev.	FJ534010	FJ533918	FJ534103	FLAS	Jacono 183	USA: FL: Franklin
045	*Marsilea vestita* Hook. & Grev.	FJ534011	FJ533919	FJ534104	FLAS	Jacono 504	USA: FL: Franklin
046	*Marsilea vestita* Hook. & Grev.	HQ631078	HQ631270	FJ534105	FLAS	Hall 414	USA: FL: Hillsborough
047	*Marsilea vestita* Hook. & Grev.	HQ631079	FJ533920	FJ534106	TENN	D. Thomas & E. Sundell 167474	USA: AR: Lincoln
048	*Marsilea vestita* Hook. & Grev.	FJ534013	FJ533921	FJ534107	TENN	D. Thomas 114172	USA: LA: Caldwell
049	*Marsilea vestita* Hook. & Grev.	FJ534014	FJ533922	FJ534108	TENN	D. Thomas & C. M. Allen	USA: LA: St. James
050	*Marsilea vestita* Hook. & Grev.	FJ534015	FJ533923	FJ534109	TENN	J. Taylor 29759	USA: OK: Johnston
051	*Marsilea macropoda* Engelm. ex A. Braun	FJ533960	FJ533868	na	TENN	G. Landry & S. Holder 7832	USA: LA: Orleans
052	*Marsilea minuta* L.	FJ533970	FJ533878	HQ631173	TENN	J.T. Beck 3867	USA: TN: Hamilton
053	*Marsilea macropoda* Engelm. ex A. Braun	FJ533961	FJ533869	FJ534052	USF	K. Bradley 1858	USA: FL: Collier
054	*Marsilea macropoda* Engelm. ex A. Braun	FJ533962	FJ533870	FJ534053	USF	B. McAlpin sn	USA: FL: Sarasota
055	*Marsilea macropoda* Engelm. ex A. Braun	FJ533963	FJ533871	FJ534054	USF	B. McAlpin sn	USA: FL: Collier
056	*Marsilea vestita* Hook. & Grev.	FJ534016	HQ631271	HQ631174	USF	A. Gholson et al. 11253	USA: FL: Franklin
057	*Marsilea minuta* L.	FJ533971	FJ533879	HQ631175	USF	J. Burkhalter 13304	USA: FL: Escambia
058	*Marsilea minuta* L.	FJ533972	FJ533880	FJ534063	USF	Jacono 667	USA: FL: Brevard
059	*Marsilea hirsuta* R. Br.	HQ631080	HQ631272	FJ534048	USF	J. Beckner 2619	USA: FL: Pinellas
060	*Marsilea hirsuta* R. Br.	FJ533955	FJ533863	HQ631176	USF	R. Wunderlin et al. 10365	USA: FL: Pinellas
063	*Marsilea mutica* Mett.	FJ533990	FJ533898	FJ534083	ASU	D. Damrel 2429	USA: AZ: Maricopa
064	*Marsilea hirsuta* R. Br.	FJ533991	FJ533899	FJ534084	ASU	L. Makings 2090	USA: AZ: Maricopa
066	*Marsilea vestita* Hook. & Grev.	FJ534017	FJ533924	FJ534110	ASU	R. Felger 85-484	Mexico: Sonora

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
067	*Marsilea deflexa* A. Braun	FJ534018	FJ533925	FJ534111	ASU	Steinman et al. 93-373	Mexico: Sonora
068	*Marsilea vestita* Hook. & Grev.	FJ534019	FJ533926	FJ534112	ASU	J. Rebman & H. Lopez 2608	Mexico: Baja California
069	*Marsilea vestita* Hook. & Grev.	FJ534020	FJ533927	FJ534113	ASU	S.Friedman&K.Johnson456-94	Mexico: Sonora
070	*Marsilea mollis* B.L. Rob. & Fernald	FJ533976	FJ533884	FJ534067	ASU	G. Ferguson 118	Mexico: Chihuahua
071	*Marsilea mexicana* A. Braun	HQ631081	HQ631273	FJ534056	ASU	J. Hernandez s.n.	Mexico: Zacatecas
072	*Marsilea fournieri* C. Chr.	FJ533952	FJ533860	FJ534045	ASU	J. Rebman & C. Davis 1684	Mexico: Baja California Sur
073	*Marsilea vestita* Hook. & Grev.	FJ534021	FJ533928	FJ534114	ASU	E. Wise 1716	USA: CA: San Luis Obisbo
074	*Marsilea vestita* Hook. & Grev.	FJ534022	FJ533929	FJ534115	ASU	R. Worthington 21894	USA: NM: Luna
075	*Marsilea vestita* Hook. & Grev.	FJ534023	FJ533930	FJ534116	ASU	D. Keil 18024	USA: CA: San Luis Obisbo
076	*Marsilea vestita* Hook. & Grev.	FJ534024	FJ533931	FJ534117	ASU	L. McGill 7203	USA: AZ: Cochise
077	*Marsilea mollis* B.L. Rob. & Fernald	HQ631082	HQ631274	FJ534118	ASU	W. T. Johnson s.n.	USA: AZ: Coconino
078	*Marsilea vestita* Hook. & Grev.	FJ534025	FJ533932	FJ534119	ASU	G. Marrs-Smith 1211	USA: AZ: Cochise
079	*Marsilea mollis* B.L. Rob. & Fernald	FJ534026	FJ533933	FJ534120	ASU	D. Damrel 627-A	USA: AZ: Gila
080	*Marsilea mollis* B.L. Rob. & Fernald	FJ534027	FJ533934	FJ534121	ASU	T. Wright & M. Baker 93-102	USA: AZ: Yaupai
081	*Marsilea mollis* B.L. Rob. & Fernald	HQ631083	HQ631275	FJ534068	ASU	J. Collins s.n.	USA: AZ: Cochise
082	*Marsilea mollis* B.L. Rob. & Fernald	FJ533977	FJ533885	FJ534069	ASU	E. Lehto 24541	USA: AZ: Coconino
083	*Marsilea mollis* B.L. Rob. & Fernald	FJ533978	FJ533886	FJ534070	ASU	M. Baker 8595	USA: AZ: Coconino
084	*Marsilea mollis* B.L. Rob. & Fernald	FJ533979	FJ533887	FJ534071	ASU	L. McGill 6860	USA: AZ:Cochise
085	*Marsilea mollis* B.L. Rob. & Fernald	FJ533980	FJ533888	FJ534072	ASU	M. Windham 0114D	USA: AZ: Coconino
086	*Marsilea mollis* B.L. Rob. & Fernald	FJ533981	FJ533889	FJ534073	ASU	D.J. Pinkava et al. s.n.	USA: AZ:Coconino
087	*Marsilea vestita* Hook. & Grev.	FJ534006	FJ533914	FJ534099	VPI	R.D. Thomas 114754	USA: LA: Caldwell
088	*Marsilea mutica* Mett.	FJ533992	FJ533900	FJ534085	VPI	M. Robinette sn	USA: VA: Patrick
089	*Marsilea vestita* Hook. & Grev.	FJ534029	FJ533936	FJ534123	ID	C.R. Bjork 6868	USA: ID: Nez Perce
090	*Marsilea vestita* Hook. & Grev.	FJ534030	FJ533937	FJ534124	ID	C.R. Bjork s.n.	USA: ID: Nez Perce
091	*Marsilea vestita* Hook. & Grev.	FJ534031	FJ533938	FJ534125	ID	A. Tiehm 15267	USA: NV: Washoe

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
092	*Marsilea vestita* Hook. & Grev.	FJ534032	FJ533939	FJ534126	ID	C.R. Bjork 2404	USA: OR: Malheur
093	*Marsilea oligospora* Goodd.	FJ534000	FJ533908	FJ534094	ID	C.R. Bjork 3916	USA: ID: Owyhee
094	*Marsilea oligospora* Goodd.	FJ534001	FJ533909	FJ534095	ID	F.D. Johnson sn	USA: ID: Idaho
095	*Marsilea polycarpa* Hook. & Grev.	FJ534002	FJ533910	FJ534096	MO	S.R. Hall & L. Phillipe 28868	Dominica: St. Andrews
096	*Marsilea vestita* Hook. & Grev.	FJ534033	FJ533940	FJ534127	UTC	N. & P. Holmgren	USA: NV: Elko
097	*Marsilea vestita* Hook. & Grev.	FJ534034	FJ533941	FJ534128	UTC	A. Tiehm 14569	USA: NV: Elko
098	*Marsilea vestita* Hook. & Grev.	FJ534035	FJ533942	FJ534129	UTC	R.J. Shaw 3708	USA: UT: Millard
099	*Marsilea vestita* Hook. & Grev.	FJ534036	FJ533943	FJ534130	UTC	Weber & Wittmann 18558	USA: CO: Baca
100	*Marsilea vestita* Hook. & Grev.	FJ534037	FJ533944	FJ534131	UTC	R.J. & M. Shaw 4986	USA: MT: Lake
101	*Marsilea minuta* L.	FJ533973	FJ533881	FJ534064	MISS	A.R. Diamond 14269	USA: AL: Pike
102	*Marsilea minuta* L.	FJ533974	FJ533882	FJ534065	MICH	D.M. Johnson 800	Trinidad: Nariva Cocal
103	*Marsilea deflexa* A. Braun	FJ533951	FJ533859	FJ534044	MICH	D.M. Johnson 794	Venezuela: Guarico
104	*Marsilea ancylopoda* A. Braun	FJ533949	FJ533856	FJ534042	MICH	D.M. Johnson 773	Argentina: Corrientes
105	*Marsilea ancylopoda* A. Braun	HQ631084	FJ533857	HQ631177	MICH	E. Lott & A. Sanders 3987	Mexico: Jalisco: Quemaro
106	*Marsilea minuta* L.	FJ533975	FJ533883	FJ534066	MICH	M. Dyer 173	Nigeria: Kano: Acha Lafia
107	*Marsilea polycarpa* Hook. & Grev.	FJ534003	FJ533911	HQ631178	MICH	W. Wagner	Puerto Rico: Loiza
108	*Marsilea polycarpa* Hook. & Grev.	FJ534004	FJ533912	FJ534097	MICH	D.M. Johnson 793	Venezuela: Apure
109	*Marsilea mollis* B.L. Rob. & Fernald	FJ533982	FJ533890	FJ534074	MICH	N. Murray & D.M. Johnson 1404	Mexico: Chiapas
110	*Marsilea vestita* Hook. & Grev.	FJ534038	FJ533945	FJ534132	MICH	B. Ertter et al. 8131	USA: CA: Contra Costa
111	*Marsilea macropoda* Engelm. ex A. Braun	FJ533964	FJ533872	FJ534055	MICH	D.M. Johnson 721	USA: TX: Aransas
112	*Marsilea coromandelina* Willd.	FJ533950	FJ533858	FJ534043	MICH	M. Dyer 172	Nigeria: Kano: Acha Lafia
116	*Marsilea mutica* Mett.	HQ631085	FJ533946	FJ534133	VPI	R. Page s.n.	USA: VA: Hanover
119	*Marsilea nashii* Underw.	HQ631086	HQ631276	HQ631179	FLAS	R.J. Abbott 8678	USA: FL: Dade
121	*Marsilea quadrifolia* L.	HQ631087	HQ631277	HQ631180	FLAS	C. Jacono 622	USA: PA: Bradford
122	*Marsilea polycarpa* Hook. & Grev.	HQ631088	HQ631278	HQ631181	FLAS	R. Dressler 6004	Panama: Panama

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
124	*Marsilea costulifera* D.L. Jones	HQ631089	HQ631279	HQ631182	FLAS	M. Whitten 3756	Australia: NSW
125	*Marsilea villosa* Kaulf.	HQ631090	HQ631280	HQ631183	BISH	Marian Chau 002	USA: HI: Oahu
127	*Marsilea villosa* Kaulf.	HQ631091	HQ631281	HQ631184	BISH	Marian Chau 029	USA: HI: Oahu
128	*Marsilea villosa* Kaulf.	HQ631092	HQ631282	HQ631185	BISH	Marian Chau 022	USA: HI: Moloka'i
129	*Marsilea villosa* Kaulf.	HQ631093	HQ631283	HQ631186	BISH	Marian Chau 015	USA: HI: Moloka'i
130	*Marsilea angustifolia* R. Br.	HQ631094	HQ631284	HQ631187	MEL	I.D. Cowie 9345	Australia: NT
131	*Marsilea crenata* C. Presl	HQ631095	HQ631285	HQ631188	MEL	G. Wightman 1458	Australia: NT
132	*Marsilea costulifera* D.L. Jones	HQ631096	HQ631286	HQ631189	MEL	I. Tankard s.n.	Australia: UC
133	*Marsilea exarata* A. Braun	HQ631097	HQ631287	HQ631190	MEL	P.K. Latz 11823	Australia: NT
134	*Marsilea exarata* A. Braun	HQ631098	HQ631288	HQ631191	MEL	M.E. Trudgen 15576	Australia: WA
135	*Marsilea exarata* A. Braun	HQ631099	HQ631289	HQ631192	MEL	P.I. Forster 20421	Australia: QLD
136	*Marsilea hirsuta* R. Br.	HQ631100	HQ631290	HQ631193	MEL	K. Alcock s.n.	Australia: SA
137	*Marsilea hirsuta* R. Br.	HQ631101	HQ631291	HQ631194	MEL	P.I. Forster 20681	Australia: QLD
138	*Marsilea hirsuta* R. Br.	HQ631102	HQ631292	HQ631195	MEL	I.D. Cowie 9590	Australia: NT
139	*Marsilea mollis* B.L. Rob. & Fernald	HQ631103	HQ631293	HQ631196	CAS	M.L. Arreguin 589	Mexico
141	*Marsilea minuta* L.	HQ631104	HQ631294	HQ631197	CAS	D.M. Johnson 800	Trinidad: Nariva Cocal
144	*Marsilea ancylopoda* A. Braun	HQ631105	HQ631295	HQ631198	CAS	E.J. Lott 3987 with A.C. Sanders	Mexico: Jalisco: Mpio La Huerta
145	*Marsilea mucronata* A. Braun	HQ631106	HQ631296	HQ631199	CAS	B. Ertter 3894 with J. Strachan	USA: CA: Plumans
146	*Marsilea vestita* Hook. & Grev.	HQ631107	HQ631297	HQ631200	CAS	A. Day 83-59	USA: CA: Merced
147	*Marsilea vestita* Hook. & Grev.	HQ631108	HQ631298	HQ631201	CAS	L. Ahart 4404	USA: CA: Sutter
148	*Marsilea vestita* Hook. & Grev.	HQ631109	HQ631299	HQ631202	CAS	A. Tiehm 11938	USA: NV: Elko
149	*Marsilea quadrifolia* L.	na	HQ631300	HQ631203	CAS	B. Bartholomew et al. Guizhou Bot. Exped. 2380	China: Guizhou
150	*Marsilea polycarpa* Hook. & Grev.	na	HQ631301	HQ631204	CAS	W.H. Wagner 82018	Puerto Rico: Loiza
151	*Marsilea oligospora* Goodd.	HQ631110	HQ631302	HQ631205	CAS	J.T. Howell 36949	USA: CA: Plumas
152	*Marsilea oligospora* Goodd.	HQ631111	HQ631303	HQ631206	CAS	A. Tiehm 13199 with G. Schoolcraft	USA: NV: Washoe

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
154	*Marsilea oligospora* Goodd.	HQ631112	HQ631304	HQ631207	JEPS	V.H. Oswald & L. Ahart 9591	USA: CA: Modoc
155	*Marsilea oligospora* Goodd.	HQ631113	HQ631305	HQ631208	JEPS	V.H. Oswald & L. Ahart 9597	USA: CA: Modoc
156	*Marsilea vestita* Hook. & Grev.	HQ631114	HQ631306	HQ631209	JEPS	L. Ahart 13088	USA: CA: Butte
157	*Marsilea vestita* Hook. & Grev.	HQ631115	HQ631307	HQ631210	JEPS	L. Ahart 14483	USA: CA: Butte
158	*Marsilea vestita* Hook. & Grev.	HQ631116	HQ631308	HQ631211	JEPS	L. Ahart 14579	USA: CA: Yuba
159	*Marsilea vestita* Hook. & Grev.	HQ631117	HQ631309	HQ631212	JEPS	L. Ahart 9320	USA: CA: Plumas
160	*Marsilea vestita* Hook. & Grev.	HQ631118	HQ631310	HQ631213	UC	B. Ertter 9696	USA: CA: Amador
161	*Marsilea oligospora* Goodd.	HQ631119	HQ631311	HQ631214	UC	V.H. Oswald &L. Ahart 4837	USA: CA: Butte
162	*Marsilea oligospora* Goodd.	HQ631120	HQ631312	HQ631215	UC	V.H. Oswald &L. Ahart 5126	USA: CA: Lassen
165	*Marsilea ancylopoda* A. Braun	HQ631121	HQ631313	HQ631216	UC	R. B. Hayward et al. 56	Venezuela: Zulia
167	*Marsilea costulifera* D.L. Jones	HQ631122	HQ631314	HQ631217	UC	R.G. Coveny et al. 12653	Australia: NSW
169	*Marsilea ancylopoda* A. Braun	HQ631123	HQ631315	HQ631218	UC	D.M. Johnson 769	Argentina: Corrientes
170	*Marsilea ancylopoda* A. Braun	HQ631124	HQ631316	HQ631219	UC	E.J. Lott 3987 with A.C. Sanders	Mexico: Jalisco
171	*Marsilea vestita* Hook. & Grev.	HQ631125	HQ631317	HQ631220	UC	A.C. Sanders et al. 13527	Mexico: Sonora
172	*Marsilea quadrifolia* L.	HQ631126	HQ631318	HQ631221	UC	B. Dickore 13374	Pakistan
173	*Marsilea vestita* Hook. & Grev.	HQ631127	HQ631319	HQ631222	UC	A. Tiehm 12601	USA: NV: Washoe
174	*Marsilea ancylopoda* A. Braun	HQ631128	HQ631320	HQ631223	UC	M. Lehnert 745	Bolivia: Tarija
175	*Marsilea deflexa* A. Braun	HQ631129	HQ631321	HQ631224	UC	R. Rueda & R. Dolmus 1221	Nicaragua
176	*Marsilea vestita* Hook. & Grev.	HQ631130	HQ631322	HQ631225	CICY	J.L. Tapia et al. 1597	Mexico: Yucatan
177	*Marsilea ancylopoda* A. Braun	HQ631131	HQ631323	HQ631226	F (US)	S. Llatas & Quiroz 2401	Peru: San Nicolas
179	*Marsilea vestita* Hook. & Grev.	HQ631132	HQ631324	HQ631227	US	J. Pruski 3743	USA: LA: Orleans
182	*Marsilea vestita* Hook. & Grev.	HQ631133	HQ631325	HQ631228	US	H. van der Werff 8708	USA: CA: San Diego
186	*Marsilea exarata* A. Braun	HQ631134	HQ631326	HQ631229	US	A. Faden 1/91	Australia: QLD
187	*Marsilea mollis* B.L. Rob. & Fernald	HQ631135	HQ631327	HQ631230	QCA	Terneus & Gonzalez 347	Ecuador: Imbabura: Lago San Pablo
188	*Marsilea mollis* B.L. Rob. & Fernald	HQ631136	HQ631328	HQ631231	GH	R. Moran 28429	Mexico: Baja California
189	*Marsilea vestita* Hook. & Grev.	HQ631137	HQ631329	HQ631232	GH	P.H. Raven 16601	USA: CA: Stanislaus
190	*Marsilea vestita* Hook. & Grev.	HQ631138	HQ631330	HQ631233	GH	G. Carnevali et al. 6740	Mexico: Yucatan

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
192	*Marsilea ancylopoda* A. Braun	HQ631139	HQ631331	na	GH	V. Solis Neffa 001	Argentina: Corrientes
194	*Marsilea tenuifolia* Engelm. ex Kunze	HQ631140	HQ631332	HQ631235	OWU	D.M. Johnson 2124	USA: TX: Mason
195	*Marsilea vestita* Hook. & Grev.	HQ631141	HQ631333	HQ631236	OWU	D.M. Johnson 2123	USA: LA: LaSalle Parish
196	*Marsilea ancylopoda* A. Braun	HQ631142	HQ631334	HQ631237	NY	N.A. Murray & D.M. Johnson 1458	Mexico: Nayarit
197	*Marsilea vestita* Hook. & Grev.	HQ631143	HQ631335	HQ631238	NY	W.T. Barker 1836	USA: KS: Kiowa
199	*Marsilea oligospora* Goodd.	HQ631144	HQ631336	HQ631239	NY	D. Lytjen 131	USA: OR: Malheur
200	*Marsilea vestita* Hook. & Grev.	HQ631145	HQ631337	HQ631240	NY	K. Thorne & S. Goodrich 3550	USA: UT: Uintah
201	*Marsilea vestita* Hook. & Grev.	HQ631146	HQ631338	HQ631241	NY	A. Tiehm 11766	USA: NV: Pershing
203	*Marsilea vestita* Hook. & Grev.	HQ631147	HQ631339	HQ631242	NY	N.H. & P.K. Holmgren 9585	USA: OR: Lake
204	*Marsilea polycarpa* Hook. & Grev.	HQ631148	HQ631340	HQ631243	NY	M. Boudrie & S. Gonzalez 4191	French Guyana
205	*Marsilea minuta* L.	HQ631149	HQ631341	HQ631244	NY	D.M. Johnson 798	Trinidad
207	*Marsilea exarata* A. Braun	HQ631150	HQ631342	HQ631245	NY	F.J. Badman 6949	Australia: SA
208	*Marsilea hirsuta* R. Br.	HQ631151	HQ631343	HQ631246	NY	C.R. Michell & D.S. Calliss 994	Australia: NT
211	*Marsilea crenulata* Desv.	HQ631152	HQ631344	HQ631247	NY	J.E. Madsen 6013	Burkina Faso: Oudalan
216	*Marsilea vestita* Hook. & Grev.	HQ631153	HQ631345	HQ631248	NY	L. Ahart 2376	USA: CA: Butte
217	*Marsilea vestita* Hook. & Grev.	HQ631154	HQ631346	HQ631249	NY	N.H. & P.K. Holmgren 14745	USA: NV: Elko
220	*Marsilea vestita* Hook. & Grev.	HQ631155	HQ631347	HQ631250	NY	R. Moran 28429	Mexico: BC
222	*Marsilea vestita* Hook. & Grev.	HQ631156	HQ631348	HQ631251	NY	Bisse et al. 32886	Cuba
224	*Marsilea uncinata* A. Braun	HQ631157	HQ631349	HQ631252	NY	S.W. Leonard et al. 8495	USA: FL: Franklin
225	*Marsilea vestita* Hook. & Grev.	HQ631158	HQ631350	HQ631253	NY	A. Taye 4957	USA: UT: Piute
227	*Marsilea mollis* B.L. Rob. & Fernald	HQ631159	HQ631351	HQ631254	NY	R. McVaugh 16917	Mexico: Jalisco
230	*Marsilea deflexa* A. Braun	HQ631160	HQ631352	HQ631255	NY	J.T. Mickel 2856	Costa Rica: Guanacaste
231	*Marsilea deflexa* A. Braun	HQ631161	HQ631353	HQ631256	NY	R. McVaugh 19287	Mexico: Nayarit
233	*Marsilea vestita* Hook. & Grev.	HQ631162	HQ631354	HQ631257	TX	W.R. Carr 25313	USA: TX: McMullen
234	*Marsilea vestita* Hook. & Grev.	HQ631163	HQ631355	HQ631258	TX	L.L. Hansen 5163	USA: TX: Coryell
235	*Marsilea macropoda* Engelm. ex A. Braun	HQ631164	HQ631356	HQ631259	TX	W.R. Carr 24546	USA: TX: Goliad
239	*Marsilea ephippiocarpa* Alston	HQ631165	HQ631357	HQ631260	AA	PRE 99559	Namibia

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
240	*Marsilea crenata* C. Presl	HQ631166	HQ631358	HQ631261	FLAS	Whitten 3765	Thailand
241	*Marsilea scalaripes* D.M. Johnson	HQ631167	HQ631359	HQ631262	FLAS	Whitten 3766	Thailand
242	*Marsilea vestita* Hook. & Grev.	HQ631168	HQ631360	HQ631263	MEXU	R.S. Felger 85-588	Mexico: Sonora
243	*Marsilea vestita* Hook. & Grev.	HQ631169	HQ631361	HQ631264	MEXU	R.S. Felger 01-746	Mexico: Sonora
244	*Marsilea vestita* Hook. & Grev.	HQ631170	HQ631362	HQ631265	MEXU	R.S. Felger 85-1118	Mexico: Sonora
256	*Marsilea aegyptica* Willd.	DQ643291	DQ643359	DQ536323	BM	Smith 3623	Namibia
257	*Marsilea ancylopoda* A. Braun	DQ643292	DQ643360	DQ536324	F	Pryer et al. 963	Puerto Rico
258	*Marsilea angustifolia* R. Br.	DQ643293	DQ643361	DQ536325	UC	Hoshizaki 1250	Australia
259	*Marsilea botryocarpa* Ballard	DQ643294	DQ643362	DQ536326	UC	Faden s.n.	Kenya
260	*Marsilea capensis* A. Braun	DQ643295	DQ643363	DQ536327	DUKE	Ramberg s.n.	Botswana
261	*Marsilea crenata* C. Presl	DQ643296	DQ643364	DQ536328	DUKE	Kato J-38	Indonesia
262	*Marsilea crenata* C. Presl	DQ643297	DQ643365	DQ536329	DUKE	Kato s.n.	Thailand
263	*Marsilea crotophora* D.M. Johnson	DQ643298	na	DQ536330	H	Ritter et al. 4561	Bolivia
264	*Marsilea distorta* A. Braun	na	na	DQ536331	BM	Kornas 6271	Nigeria
265	*Marsilea drummondii* A. Braun	DQ643299	DQ643366	DQ536332	UC	Hoshizaki 577	Australia
266	*Marsilea ephippiocarpa* Alston	DQ643300	na	na	UC	Chase 2255	Zimbabwe
267	*Marsilea fadeniana* Launert	DQ643301	DQ643367	DQ536333	US	Evans and Maikweki 55	Kenya
268	*Marsilea farinosa* Launert	DQ643302	DQ643368	DQ536334	US	Faden 70/902	Kenya
269	*Marsilea gibba* A. Braun	DQ643303	DQ643369	DQ536335	US	Faden and Ng'weno 87/33	Kenya
270	*Marsilea macrocarpa* C. Presl	DQ643304	DQ643370	DQ536336	UC	Hoshizaki 236	South Africa
271	*Marsilea macropoda* Engelm. ex A. Braun	DQ643305	DQ643371	DQ536337	DUKE	Hoshizaki 1458	USA: TX
272	*Marsilea minuta* L.	DQ643306	DQ643372	DQ536338	DUKE	Shimozono s.n.	Burma
273	*Marsilea minuta* L.	DQ643307	na	DQ536339	DUKE	Rajesh 87938	India
274	*Marsilea minuta* L.	DQ643308	DQ643373	DQ536340	DUKE	Hoshizaki 237	Africa
275	*Marsilea mollis* B.L. Rob. & Fernald	na	na	DQ536341	F	Johnson s.n.	Mexico
276	*Marsilea mutica* Mett.	DQ643309	DQ643374	DQ536342	DUKE	Hoshizaki 840	New Caledonia
277	*Marsilea nashii* Underw.	DQ643311	DQ643376	DQ536344	DUKE	Correll s.n.	West Indies
278	*Marsilea nashii* Underw.	DQ643310	DQ643375	DQ536343	F	Correll 46631	Turks & Caicos

TABLE 1. Continued.

DNA sample number	Taxon	rbcL	trnL-F	rps4	Herbarium of deposition	Voucher	Locality
279	*Marsilea nubica* var. *gymnocarpa* (A. Braun) Launert	DQ643312	na	DQ536345	BM	Smith 1988	Botswana
280	*Marsilea nubica* var. *gymnocarpa* (A. Braun) Launert	DQ643313	na	DQ536346	BM	Kornas 6379	Nigeria
281	*Marsilea oligospora* Goodd.	DQ643314	DQ643377	DQ536347	UC	Tiehm 13199	USA: NV
282	*Marsilea polycarpa* Hook. & Grev.	DQ643315	DQ643378	DQ536348	DUKE	Pryer 960	Puerto Rico
283	*Marsilea quadrifolia* L.	DQ643316	DQ643379	DQ536349	DUKE	Anno s.n.	Japan
284	*Marsilea schelpeana* Launert	DQ643317	DQ643380	DQ536350	DUKE	Honshizaki 742	South Africa
285	*Marsilea vera* Launert	DQ643318	na	DQ536351	BM	Burrows 3716	Botswana
286	*Marsilea vestita* Hook. & Grev.	DQ643319	DQ643381	DQ536352	US	Howell 47460	USA: CA
287	*Marsilea villifolia* Brem. & Oberm. ex Alston & Schelpe	DQ643320	na	DQ536353	BM	Hansen 3232	Botswana
288	*Marsilea villosa* Kaulf.	DQ643321	DQ643382	DQ536354	US	Degener 9049	USA: HI
289	*Pilularia americana* A. Braun	DQ643288	DQ643383	DQ536355	DUKE	Pryer 978	USA: GA

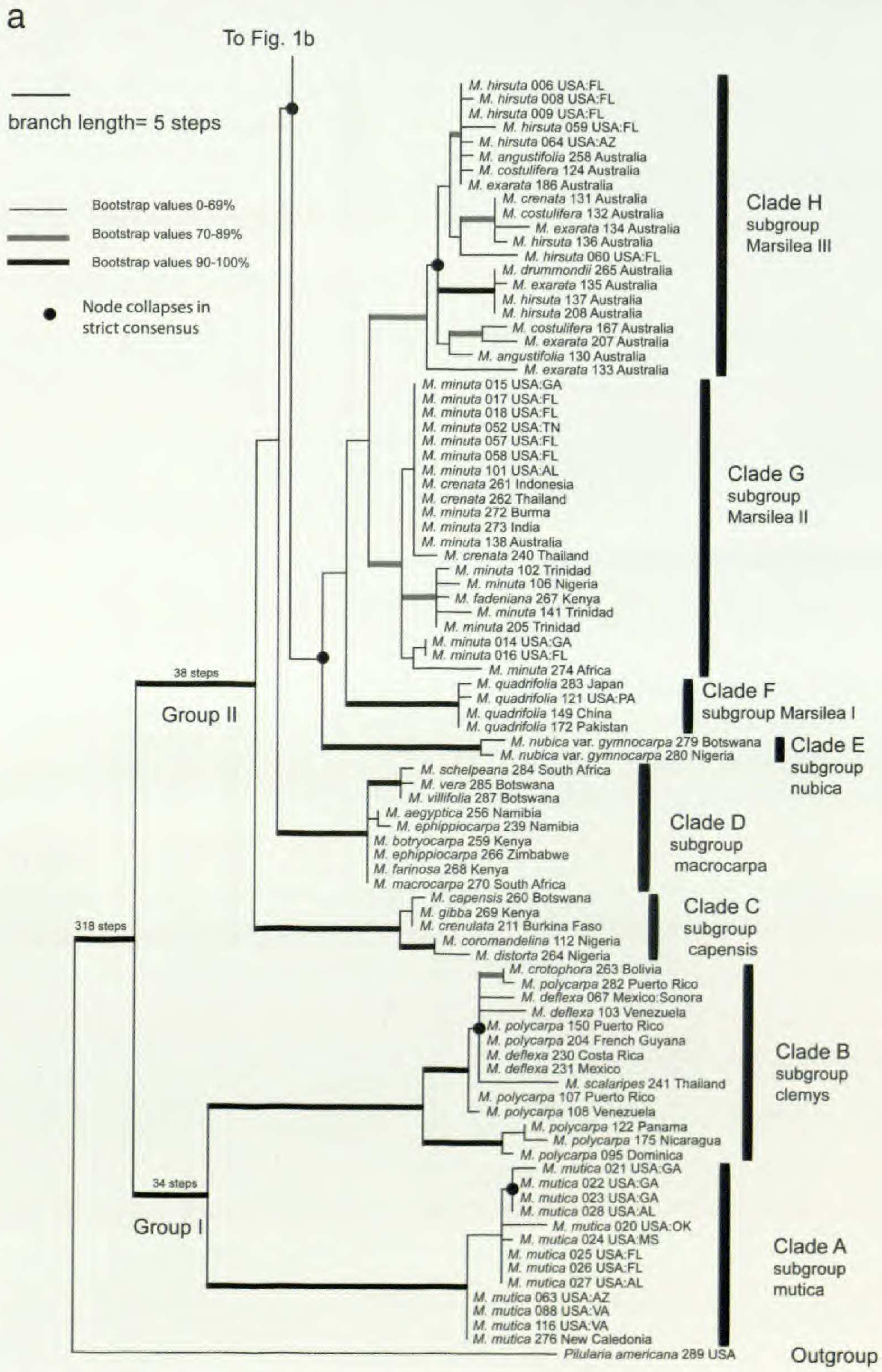

FIG. 1. (A–C). A single randomly-chosen shortest tree from maximum parsimony analysis of *Marsilea* combined plastid DNA data matrix (*rbcL*, *rps4*, *rps4-trnS* spacer, and *trnL-F* spacer). Branch lengths are indicated by scale bars (except for longer branches of Fig. 1a); bootstrap support is indicated by branch thickness/grayscale. Nodes that collapse in the strict consensus are marked with a black dot. Tree length = 743; consistency index (CI) = 0.80; retention index (RI) = 0.96. Major clades are labeled A–L; the informal clade names (groups and subgroups) correspond to those used in Nagalingum *et al.* (2007).

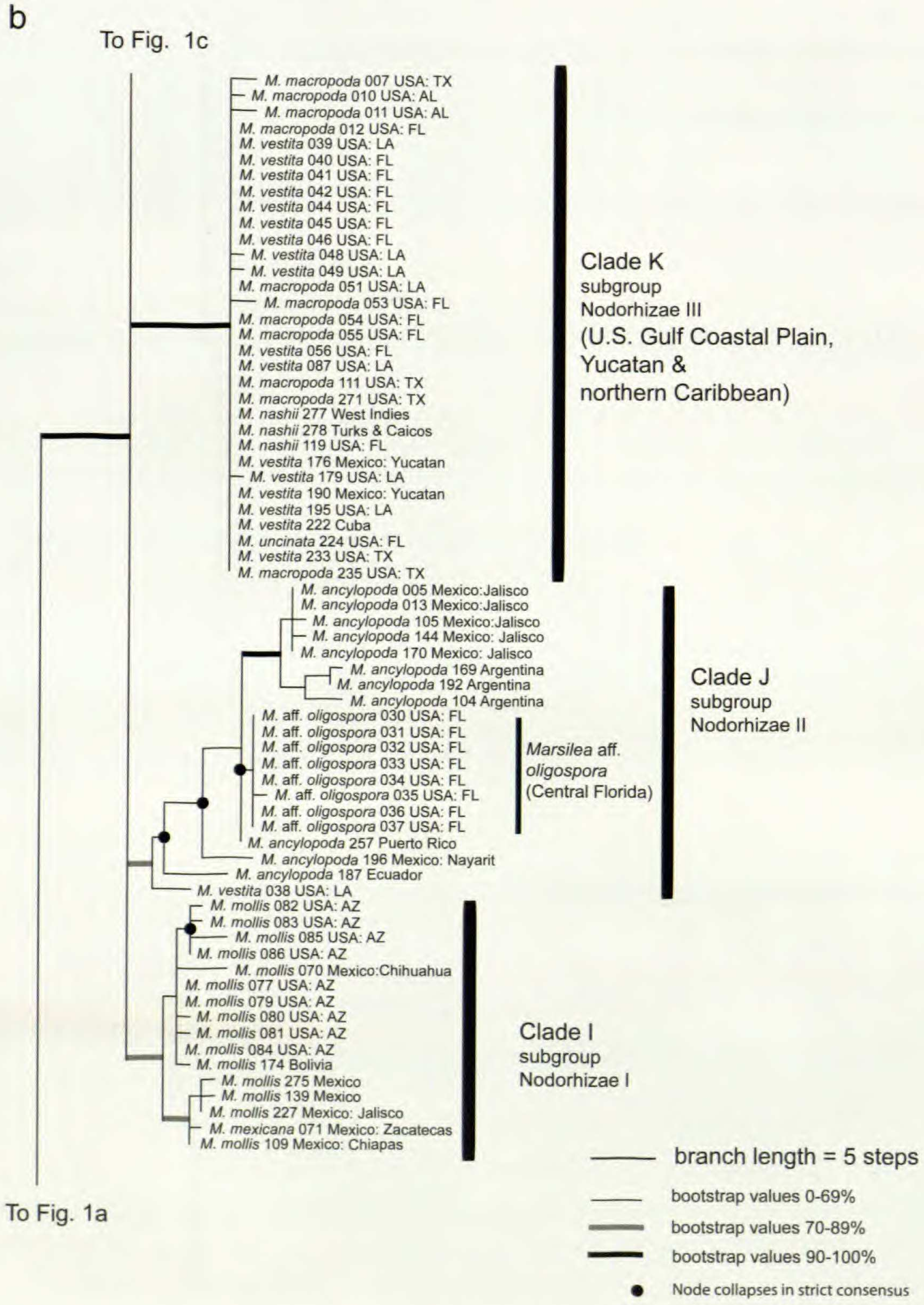

FIG. 1 Continued.

pieces, facilitating amplification from degraded total DNAs. Primers for *rbcL* are: *rbcL*F ATGTCACCACAAACAGAGACTAAAGC; *rbcL* intF TGAGAACG-TAAACTCCCAACCATTCA; *rbcL* intR CTGTCTATCGATAACAGCATGCAT; and *rbcL*R GCAGCAGCTAGTTCCGGGCTCCA. The *rps4* exon and the adjacent

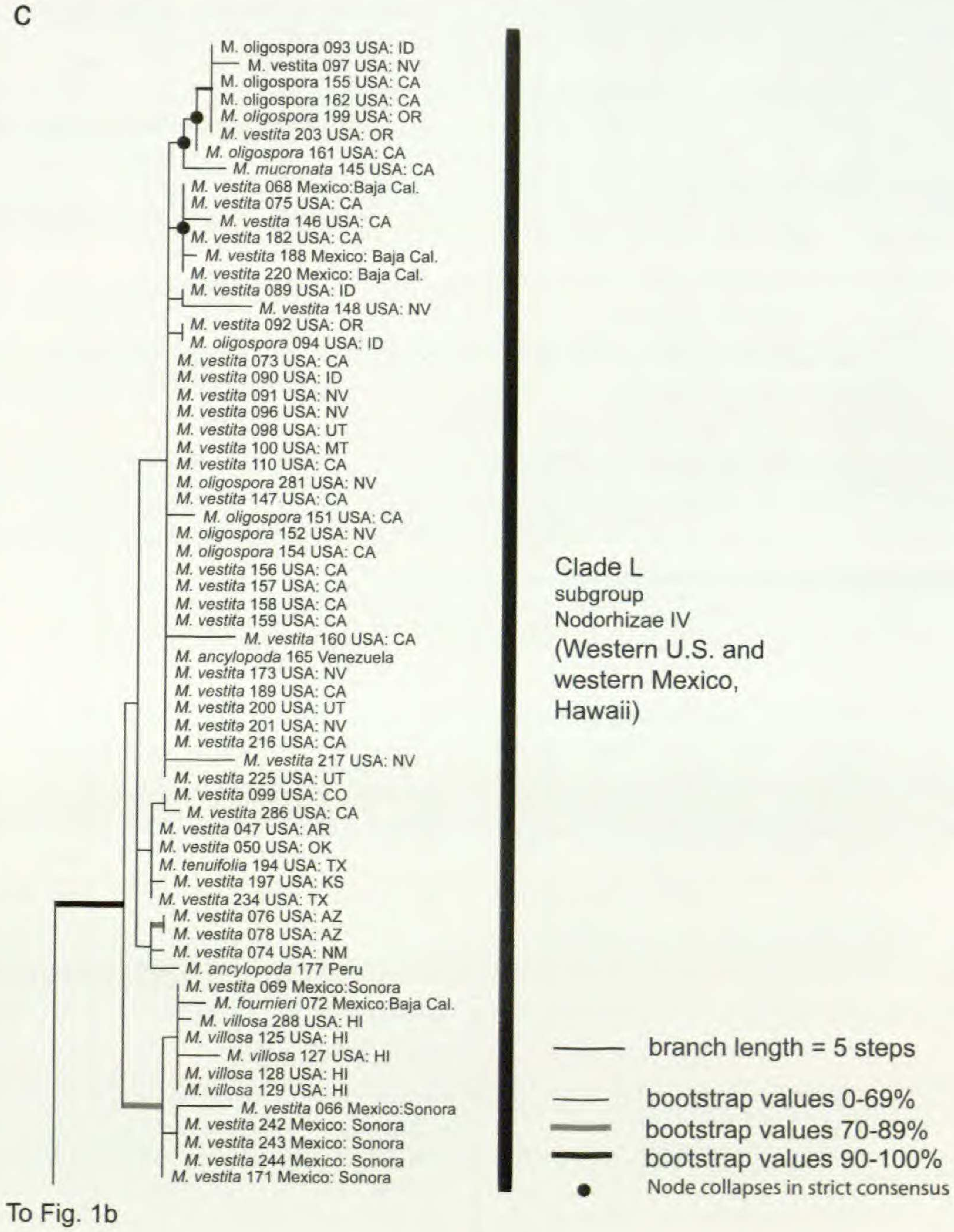

FIG. 1 Continued.

rps4-trnS spacer were amplified in one piece using the primers *rps4*F ATGTCCCGTTATCGAGGACCT and *rps4*R TACCGAGGGTTCGAATC; problematic samples were amplified in two pieces using the internal primers *rps4* intF TGCCAAACGAGAATCTATGG and *rps4* intR CGATGGGTTGT-TAGTTGTTAG. Primers for the *trnL-F* spacer (primers E&F) were those of Nagalingum *et al.* (2007). All amplifications utilized Sigma Jumpstart *Taq* polymerase and reagents (Sigma-Aldrich, Inc., St. Louis, MO, USA) in 25 μl reactions with 3.0 mM $MgCl_2$. Thermocycler conditions were: 94 °C for

3 minutes followed by 37 cycles of 94 °C for 30 s, 56 °C for 30 s, 72 °C for 2 min, with a final extension of 3 min at 72 °C. Problematic taxa were amplified using Phusion polymerase (New England Biolabs, Ipswitch, MA, USA) according to manufacturer's protocols. PCR products were sequenced in both directions using the Big Dye Terminator reagents on an 3130 automated sequencer following manufacturer's protocols (Applied Biosystems, Inc.). Electropherograms were edited and assembled using Sequencher 4.10 (GeneCodes Inc., Ann Arbor, MI, USA), and the resulting sequences were aligned manually using SE-AL (Rambaut, 1996). All sequences were deposited in GenBank (Table 1). A 25 bp portion of the *rps4-trnS* spacer contained a homopolymer region of ambiguous alignment; this region was excluded from analyses. We analyzed the data using maximum parsimony rather than maximum likelihood because the number of steps in the resulting trees more clearly represents the number of base pair differences among accessions. Analyses were performed using PAUP* version 4.0 b10 (Swofford, 2003) with Fitch parsimony (equal weights, unordered characters, ACCTRAN optimization and gaps treated as missing data). Heuristic searches consisted of 1000 random taxon addition replicates of subtree-pruning-regrafting (SPR) and "keeping multiple trees" (MULTREES) with the number of trees limited to 10 per replicate to minimize extensive swapping on islands with many suboptimal trees; 10,000 shortest trees were saved. Support was estimated by 1000 bootstrap (BS) replicates, saving only 5 trees per replicate and ten trees per bootstrap replicate. The data matrix is available from the senior author or at ftp://ftp.flmnh.ufl.edu/Public/Marsilea/.

Results

In total, our dataset comprised 2629 characters for a total of 223 ingroup accessions, plus *Pilularia americana* A.Braun. We used existing sequence data for 33 accessions from 26 species and newly sequenced data for an additional 190 accessions from 12 species (Table 1).

Figure 1 (a, b, c) presents a single randomly-chosen maximum parsimony (MP) phylogram out of 10,000 shortest trees saved. Tree length = 743; consistency index (CI) = 0.80; retention index (RI) = 0.96; ACCTRAN optimization. BS values are indicated by line thickness and shading of branches.

The DNA data revealed that several specimens sampled in this study were misdetermined (based upon their anomalous placement in the tree and reexamination of the voucher specimens). DNA data were especially effective in clarifying the identification of sterile specimens of both North American and introduced origin.

The cladogram is distinguished by a basal dichotomy separating two strongly supported clades, earlier designated informally as Groups I and II (Schneider and Pryer, 2001; Nagalingum *et al.*, 2007). Group I comprises informal subgroups "mutica"/A and "clemys"/B, and Group II includes subgroups "capensis", "macrocarpa", "nubica", "marsilea I–III", and "nodorhizae I–IV", here designated Clades C through H, respectively. Clades A and C–H are Old World (Launert, 1968) and Clades A–G have glabrous leaves. Clade H includes hairy-leafed species from Australia. Clades I, J, K, and L are New World, have hairy leaves typical of the semi-aquatic ecotype, and include the majority of the specimens sequenced in this study. These latter four clades are united by high BS support into a single clade that corresponds to Johnson's *Marsilea* sect. *Nodorhizae* (Johnson, 1986; Nagalingum *et al.*, 2007), which includes six species (plus many names that Johnson synonymized).

Clade A is monotypic, consisting only of *M. mutica* Mett. It is clearly distinct from all other taxa in terms of DNA sequence and morphology, with its two-toned leaflets and petioles inflated at the apex to function as air bladders for floating leaves. This species has elliptical sporocarps that lack a transverse vein, are borne at the base of the petiole, and are either solitary or in clusters of 2–4 on branched pedicels. Indigenous to Australia and New Caledonia, *M. mutica* may be the most popular species in the water garden trade. The southeastern U.S. specimens plus one from Oklahoma are genetically distinct from specimens from Arizona and Virginia, a result suggestive of at least two distinct geographic origins for material introduced into the U.S.

Clade B includes several species that share the distinctive feature of linear rows of globose sporocarps borne on the petiole and a transverse sporocarp veining; this clade corresponds to *Marsilea* sect. *Clemys* (Johnson, 1986, 1988). The inclusion of *M. scalaripes* and *M. deflexa* in this clade confirms their hypothesized placement in the *clemys* subgroup (Nagalingum et al., 2007). However, these plastid data do not resolve the sampled taxa into monophyletic species. There are two well-supported (between 90–100% BS) clades, both of which include samples of *M. polycarpa* Hook. & Grev. and *M. deflexa*. The non-monophyly of species in this clade and Johnson's (1986) putative designation of hybrids of these species may warrant a reexamination of determinations of these specimens and/or species concepts. Sample #175 from Nicaragua is sterile and its determination as *M. deflexa* is tentative.

Clade C contains five African species: *M. capensis* A.Braun, *M. gibba* A.Braun, *M. crenulata* Desv., *M. distorta,* and *M. coromandelina,* which as described by Launert (1968) are all of the glabrous leaflet type. Although this clade is strongly supported (100% BS), the plastid data fail to fully resolve relationships among these species.

Clade D contains eight African species: *M. schelpeana* Launert, *M. aegyptica* Willd., *M. botryocarpa* Ballard, *M. ephippiocarpa* Alston, *M. farinosa* Launert, and *M. macrocarpa* C.Presl, and partial plastid data also place *M. vera* Launert and *M. villifolia* Brem. & Oberm. ex Alston & Schelpe in this clade. In contrast to Clade C, all eight species of Clade D are of the hairy leaflet type (Launert, 1968).

Clade E consists of two samples of *M. nubica* A.Braun, a glabrous species from Africa that forms abundant colonies (Launert, 1968).

Clade F consists entirely of *M. quadrifolia* L., the type species of the genus, the only glabrous species from a cool-temperate climate, and a protected species in Europe. Four accessions from different continents, both native and introduced in range, display little sequence variation.

Clade G is moderately supported (84% BS) and includes a single accession of the African species *M. fadeniana* Launert, several Asian accessions of *M. crenata* C.Presl, and numerous accessions of *M. minuta* L., including several from introduced populations in the southeastern U.S. and Trinidad. The *M. crenata – M. minuta* complex is one of the largest and most variable groups within the genus (Launert, 1968). Earlier molecular data showed that *M. crenata* was nested within *M. minuta* (Nagalingum *et al.,* 2007), and the addition of more accessions provides additional evidence that the two taxa are likely conspecific. Three samples from Trinidad (introduced) form a moderately supported clade with samples from Kenya and Nigeria. A single accession (#138) originally determined as *M. hirsuta* was probably misdetermined, but was not available for examination.

Clade H includes Australian hairy-leaved species: *M. drummondii* A.Braun, *M. exarata*, *M. hirsuta* R.Br., *M. angustifolia* R.Br., and *M. costulifera*. There are several subclades resolved, but only one has high (90–100%) bootstrap support. None of the species within this clade are resolved as monophyletic. DNA data fail to distinguish *M. hirsuta* from *M. angustifolia*. Morphologically, *M. angustifolia* differs from *M. hirsuta* in having smaller and more elongated leaves and smaller sporocarps (Aston, 1973). These characters, however, are typically considered insufficient for species distinction within the genus (Launert, 1968). This clade includes a single specimen (#131) determined as *M. crenata*; it is probably misdetermined, as all other specimens of *M. crenata* fall in Clade G.

The majority of the specimens sampled are in Clades I, J, K, and L; these form a highly supported group that include all species native to North and South America. Species within each clade are poorly resolved due to low sequence divergence. Both clades K and L include members of a complex of mainly North American species related to *M. vestita* Hook. & Grev. and *M. oligospora*. Although they receive moderate to high bootstrap support, clades K and L correlate strongly with geographic origin (K=U.S. Gulf coastal plain, Yucatan, Mexico, and the northern Caribbean; L= Mexico, western U.S., and Hawaii), but not with accepted species concepts.

Clade I consists primarily of accessions of *M. mollis* B.L.Rob. & Fernald from north central Mexico, Arizona, and one from Bolivia. One specimen from Zacatecas, Mexico is determined as *M. mexicana*; the molecular data do not distinguish it from *M. mollis*.

Clade J has partially resolved but unsupported internal structure and includes *M.* aff. *oligospora* from Florida, *M. ancylopoda* from west-central Mexico, Puerto Rico and northeastern Argentina, plus one sterile sample (#187) originally determined as *M. mollis* from Andean Ecuador (Lago San Pablo). Johnson (1986) cited three sterile collections of *M. mollis* from this same lake and suggested that many sterile Andean collections above 1500 m are probably referable to *M. mollis*. Our molecular data indicate these Ecuadorian collections are not *M. mollis*, but instead belong to this clade that includes *M. ancylopoda*.

Sample #38 (*M. vestita* from Louisiana) is sister to all other taxa in this clade in the strict consensus of all trees; its anomalous placement caused us to resequence this specimen, but the second sequence was identical to the first.

Clade K includes specimens of *M. vestita*, *M. macropoda*, and one of *M. uncinata* from the Gulf coastal plain of the southeastern U.S., together with several accessions of *M. nashii* from Yucatan and the northern Caribbean. Johnson (1986) regarded *M. uncinata* as a synonym of *M. vestita*, but considered *M. nashii* to be a valid species distinguished by its strongly nodding sporocarps (vs. slightly nodding to ascending in *M. vestita*), a feature which we have found to vary greatly across and within species, presumably in response to the microenvironment under which sporocarps develop. The molecular data provide no resolution within this clade.

Clade L consists mostly of specimens of *M. vestita* and *M. oligospora* from central Texas through the western United States and northwestern Mexico, plus a specimen of *M. mucronata* A.Braun from California, which Johnson (1986) regarded as a synonym of *M. vestita*. It also includes two specimens of *M. ancylopoda* from Venezuela and Peru, but they are not resolved as sister taxa. The clade also includes several accessions of *M. villosa* Kaulf., an endangered Hawaiian endemic, which form a weakly supported clade with *M. vestita* and *M. fournieri,* both from Baja California, Mexico. Johnson (1986) considered *M. fournieri* C.Chr. to be a small-leaved form of *M. vestita*. This tree is consistent with the hypothesis that *M. villosa* arose via long-distance dispersal of *M. vestita* from western Mexico to the dry lowlands of Moloka'i, Ni'ihau, and O'ahu where seasonal flooding of shallow depressions offers restricted habitats (Wester, 1994). This clade also includes samples of *M. oligospora* from northern California and Idaho; the type locality of this species is in Wyoming (see discussion of *M.* aff. *oligospora* in Florida in clade J). The ten samples of *M. oligospora* are not monophyletic and are scattered throughout this clade, but without resolution or support.

Discussion

Evaluation of Florida *Marsilea* aff. *oligospora*

Based on our phylogenetic trees, the eight accessions of *M.* aff. *oligospora* from central Florida (samples 30–37) fall within Clade J; these plants form a weakly supported clade distinct from all others and are sister to *M. ancylopoda* from Mexico and Argentina. These eight plants also share a four basepair insertion in the *trnL-F* spacer that is absent in all other *Marsilea*; this indel is an unambiguous synapomorphy that distinguishes these Florida plants. Jacono and Johnson (2006) tentatively identified these populations as *M.* aff. *oligospora*, although noting subtle morphological differences from western U.S. *M. oligospora*, and they regarded the Florida populations as introductions from the western U.S. Our data contradict their hypothesis; "true" *M. oligospora* (e.g., samples 93 and 94, from near the type locality in the western U.S.; Jackson Hole, Wyoming) fall in clade L, and our data clearly distinguish the Florida populations from all other taxa. The molecular data indicate that these Florida populations are nested within *M. ancylopoda* from Mexico, Puerto Rico, Argentina, and Ecuador (based on current sampling).

According to Johnson's (1986) morphological concept of *M. ancylopoda*, the species includes considerable variation in sporocarp morphology, but the sporocarps always lack a superior tooth. The Florida populations of *M.* aff. *oligospora* (sensu Jacono and Johnson, 2006) bear sporocarps with prominent tooth. The presence of toothed and toothless taxa together in Clade J indicate that this character may be homoplasious and may not provide reliable characters for diagnosis of species, at least within this species complex.

The data show that central Florida specimens of *M.* aff. *oligospora* (samples 30–37) are distinct from all other sampled *Marsilea* and might represent an undescribed species or a morphological and molecular variant of *M. ancylopoda*. We are unable to match them with *Marsilea* from any other geographic locality. Our sampling of the Caribbean, Central America, and northern South America is poor, and more extensive sampling might provide a match for the Florida populations. The type of *M. ancylopoda* is from coastal arid lowlands just north of the Gulf of Guayaquil, Ecuador. Future studies should include material from the type locality. Although our sampling does not include material from the type locality of *M. ancylopoda*, it does include Peruvian material from similar low-lying habitats along the arid west coastal strip of South America. This specimen (#177, *Llatas & Quiroz 2401*), is in Clade L where it groups weakly with *M. vestita* from the desert regions of New Mexico and Arizona. Additional sampling from low elevation neotropical localities is also needed to seek matches for *M. ancylopoda* from west-central Mexico and northeastern Argentina, as included in Clade J of this study. Until further sampling yields a match for the Florida plants, we suggest that the populations should be regarded as endemic and given protected status by vegetation managers until its status as native or alien is resolved more definitively.

Evaluation of Morphological Species Concepts in *Marsilea* Section *Nodorhizae*

These plastid data provide an independent dataset with which to evaluate morphological species concepts in *Marsilea*, especially for the North American species that were heavily sampled. The failure of the plastid data to resolve specimens into clades that correspond to morphospecies is most obvious in *Marsilea* sect. *Nodorhizae* (*M. oligospora*, *M. mollis*, *M. villosa*, *M. vestita*, *M. macropoda*, *M. nashii*, and *M. ancylopoda*). Instead, plastid data group these seven species into four distinct clades with strong geographic structure that correspond to climactic and habitat zones: Clade L includes western North American accessions from ephemeral ponds in arid climates; Clade K includes plants from humid, seasonally influenced low elevation floodplains and wet depressions of the Gulf coastal plain, Florida, and the northern Caribbean; Clade I consists only of *M. mollis* from Arizona to Bolivia; Clade J includes *M. ancylopoda* (from Mexico and Argentina), the central Florida material (*M.* aff.

ancylopoda) and nearby Puerto Rico, plus a geographically disparate accession from the montane highlands of north central Ecuador and an aberrant sterile specimen from Louisiana (#038).

The incongruence of these plastid trees and the currently accepted species of *Marsilea* may have several explanations, which we discuss below. Extensive hybridization among *Marsilea* species might have led to chloroplast capture of a single plastid type among many species resulting in plastid trees that do not accurately reflect phylogenetic relationships. Johnson (1986) cited several specimens as putative interspecific hybrids, based solely on interpretation of subtle morphological characters. To our knowledge, no one has created artificial *Marsilea* hybrids, nor used molecular data to demonstrate the parental origin of putative hybrids. Additionally, the non-monophyly of species may be due to incomplete lineage sorting. However, we did not examine the individual gene trees to determine if this could be the cause of non-monophyly.

The absence of monophyletic species may also be due to the presence of cryptic species. This is exemplified by our finding that the plants originally identified as *M. aff. oligospora* are a potentially undescribed species (see above). These plants display subtle morphological differences compared to all other known *Marsilea*, and molecular data indicate that they have a unique molecular signature as well. Therefore, it is possible that through more intense sampling and reassessment of morphology, the non-monophyletic species may reveal the presence of underlying cryptic species.

Through our analyses we discovered several accessions that were misidentified, and it is possible that some of polyphyletic species are due to identification errors. However, given the extent of polyphyletic species (and that many specimens were annotated by D.M. Johnson), we suggest that this is unlikely.

A final explanation for failure of the existing alpha-taxonomy could be an inflated number of species within *Marsilea* sect. *Nodorhizae* (clades J, K, L). Many species of *Marsilea* are based upon subtle morphological traits that are phenotypically plastic or that represent homoplasious local adaptations to environmental conditions. We found that plant size, leaflet size, extent of leaflet hairiness, the angle and extent of sporocarp nodding, and the curvature of the peduncle demonstrated variability that might preclude their taxonomic utility for species delimitation.

It was beyond the scope of this project to re-examine all of the specimens used in this study. However, we suggest that future work include re-examination of the morphology of multiple accessions within a phylogenetic framework to ascertain the reliability of the existing characters for species delimitation and to determine if cryptic species are present. There is also the need for more extensive sampling and sequencing of more variable plastid regions, to be contrasted with nuclear gene data sets, which will provide a better framework to settle questions of hybridization and incomplete lineage sorting as well as provide greater resolution and support for the relationship among species.

Conclusions

Using the extensively sampled phylogeny, we found that Florida plants earlier identified as *M. aff. oligospora* possess a unique molecular signature (an insertion in the *trnL-F* spacer) but morphological characters that distinguish it from other taxa in *M.* sect. *Nodorhizae* are subtle and require more detailed analyses (Jacono and Johnson, 2006). It is possible that these plants represent an undescribed, cryptic species endemic to Florida, or a geographically restricted variant of an existing species. Our plastid trees reveal the same major clades as the previous study by Nagalingum *et al.* (2007). Although our increased taxon sampling reveals no conflicts, many species are not resolved as monophyletic within these informally named clades. We were unable to determine if this is due to hybridization, incomplete lineage sorting, misidentification of specimens, the presence of cryptic species, and/or inappropriate morphological characters for species delimitation—the present data are inadequate to resolve these large taxonomic questions. We advise that the existing alpha-taxonomic classification and circumscription of species in *Marsilea*, especially *M.* sect. *Nodorhizae*, should be treated with caution.

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